*ON EXCITATION OF BIOLOGICAL SUBSTANCES**

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It has been noted[1] that watery solutions of fluorescent dyes profoundly alter their optical behavior on freezing: their fluorescent emission disappears. On further lowering of the temperature, light emission reappears, but the emitted light differs from the earlier fluorescent light in having a longer wave length and coming from an excitation having a longer lifetime, answering, thus, to the formal definition of "phosphorescence." This change in properties is not limited to dyes. Certain proteins, amino acids, coenzymes, and steroid hormones, for instance, show analogous behavior.

With regard to its possible biological implications, this phenomenon deserves further study. Two questions suggest themselves: (1) Does the phosphorescent light originate from a triplet excitation or from a crystal phosphor? (2) Is this behavior a universal property of fluorescent substances? The experiments reported in this paper suggest answers to these questions.

There are several criteria which can be utilized in studies of luminescence to characterize an emission as arising from a metastable triplet level.

1. Triplet emission is shifted toward the longer wave lengths relative to the fluorescent emission.
2. Triplet emissions have a much longer lifetime than singlet emissions.

3. Triplet-state emissions obey first-order decay kinetics.
4. Triplet-state emissions are usually only slightly temperature-dependent, i.e., activation energy is negligible.
5. The molecule in the triplet state, having an unpaired electron spin, is paramagnetic.
6. Triplet emissions induced by polarized exciting light are slightly polarized or nonpolarized.
7. The molecule in the triplet state, being a chemical species in its own right with its own set of properties, will exhibit its own characteristic absorption spectrum.

For the work reported in this paper we have used Criteria 1–4 to aid in characterizing the electronic states involved.

METHODS

CRITERION 1: EMISSION SPECTRA

A. VISUAL

A knowledge of the fluorescent spectrum for a particular compound permits one to observe visually whether a subsequent delayed emission has undergone a wave-length shift. For example, tyrosine fluorescence resides exclusively in the ultraviolet, but when the compound is frozen in the presence of glucose, it exhibits, upon proper excitation, a pale blue afterglow. We recognize this shift of the emission toward longer wave lengths as presumptive evidence for the occurrence in the molecule of an electronic transition different from that responsible for the ultraviolet fluorescence. In this study most of our observations of wave-length shift have been visual.

B. INSTRUMENTAL

1. *Beckman DK-1 Spectrophotometer.*—We have occasionally measured fluorescent and phosphorescent spectra with the DK-1 spectrophotometer by a technique described by Gemmill.[2] Spectra obtained by this method are subject to considerable error unless the spectral response of the prism-photomultiplier assembly has been calibrated against a standard lamp.

2. *Leiss Monochromator–Red-sensitive Phototube Assembly.*—Emission spectra have been obtained with a Leiss fused quartz monochromator coupled with a DuMont red-sensitive photomultiplier tube, type K 1292, sensitive to 1.3 μ. The photomultiplier signal was piped into a Beckman Model V micromicroammeter, which was reversed to take a negative input signal. Spectral emission intensity was measured as a linear response of the ammeter-galvanometer deflections. The monochromator-photomultiplier-ammeter assembly was calibrated for spectral distribution intensity in relative units against a standard lamp calibrated for us by the National Bureau of Standards. Monochromator slit widths were 0.5 mm. Samples under study were contained in flat-faced Pyrex cuvettes of 4-mm. inside width and were frozen in liquid nitrogen contained in an unsilvered Pyrex Dewar flask. The frozen system was illuminated from one side with a Hanovia Compact xenon arc, and the emission from the opposite side was introduced into the entrance slit of the Leiss monochromator. Judicious use of liquid and glass filters permitted proper separation of exciting and emitted light.

CRITERION 2: LIFETIMES

Since fluorescent lifetimes are of the order of 10^{-8} second in duration, any emission which is apparent after removal of the exciting source, especially when accompanied by a shift toward longer wave lengths relative to the fluorescence emission, is indicative of a metastable-state involvement. Increased lifetimes are obvious visually and may be quantitated from kinetic studies.

CRITERION 3: DECAY KINETICS

The Beckman DK-1 automatic recording spectrophotometer was used for kinetic studies. With the instrument set to record energy, the wave-length drum was set to an appropriate wave length, and the slits were set to maximum width, namely, 2.0 mm. Aqueous solutions of the systems under study were placed in 25 × 200-mm. Pyrex test tubes and immersed initially in a methanol–dry ice bath for prior freezing. Systems studied at 194° K. were permitted to remain in this bath for a minimum of 15 minutes to attain temperature equilibrium. Samples to be studied at 77° K., following freezing in the dry-ice bath, were then transferred to Dewar vacuum flasks containing liquid nitrogen. Samples were allowed to remain here for a minimum of 15 minutes to insure temperature equilibration, following which they were placed for 5 seconds in the unfiltered beam of a Hanovia high-pressure mercury lamp. The glowing sample was then immediately transferred into the lamp-housing compartment of the DK-1 and positioned at the light-entrance port. Following an immediate deflection response of the potentiometer, the magnitude of which depended on the intensity of the afterglow, the decay was tracked automatically, with the recorder chart set to roll at 12 inches per minute. Energy recorded in this manner on the DK-1 was read off the graph in relative linear units. The curves so obtained were examined for fit to kinetic equations.

In making kinetic studies with the DK-1, it should be noted that the instrument itself has a finite decay time. For example, in recording on energy with a light source inducing a 100 per cent energy reading on the potentiometer, shutting off the light causes the potentiometer to "decay" to 0 per cent energy. By running the chart at 12 inches per minute, we were able to obtain an instrument "decay curve." For our instrument this "decay curve" was found to fit a first-order kinetic equation and had a rate constant of approximately 1. Kinetic data obtained with the DK-1, therefore, represent mixtures of two rate constants, that of the instrument and that of the sample being studied. We express our thanks to Dr. William Arnold for deriving an equation for us which permitted the two rate constants to be resolved from mixed kinetic data. Using this equation we have checked the accuracy of our experimentally determined rate constants. The reported rate constants are accurate to within ±5 per cent; most within ±2 per cent usually on the low side.

In a communication from the Beckman Instrument Company concerning the use of the DK-1 spectrophotometer for rapid kinetic studies they pointed out that one should be able to determine the time constant (reciprocal of the rate constant) for a phosphorescent decay if it is not less than 10 per cent of the time constant of the DK-1. In our studies no phosphorescences were observed with rate constants exceeding that of our DK-1.

It should be emphasized that in the text figures recording our kinetic results, the ordinates represent phosphorescent emission intensities in relative energy units. No significance is to be attached to the relative vertical positions of the curves on the logarithmic scale. The positions were selected for arbitrary convenience in plotting the data.

A statement accompanying the figures to the effect that the emission for a particular rate study was unfiltered means that the RCA 1P28 photomultiplier tube on the DK-1 was exposed *directly* (the prism system was not traversed) to the glowing sample under study. This technique was possible only at the temperature of dry ice, for at lower temperatures nonspecific emissions interfered. The technique also required extra precautions to exclude outside light from the photomultiplier tube.

CRITERION 4: ACTIVATION ENERGY

Activation energies were measured in the usual way, i.e., from the slope obtained by plotting the log of the rate constant against the reciprocal of the absolute temperature. The temperatures used were those of dry ice (194° K.) and liquid nitrogen (77° K.).

RESULTS

We have obtained and studied delayed emissions for the following compounds (source given): bullock lens, intact and homogenized; tryptophan and tyrosine, H & M Chemical Company; adenine HCl and adenosine, Schwarz Laboratories, Inc.; adenylic acid (muscle), adenosine diphosphate, and adenosine triphosphate, Pabst; desoxynucleic acid (sperm) and ribonucleic acid, Nutritional Biochemical Corporation; progesterone and alpha-estradiol, Ciba; quinine sulfate, Merck; quinidine sulfate, Fisher; acridine orange and acridine yellow, National Aniline Division, Allied Chemical and Dye Corporation. We will now consider the results for the individual materials separately.

1. BULLOCK LENS

For the study of the optical properties of structural proteins, the lens, being devoid of pigments, offers a unique material.[3] Lenses taken from different animals —bullock, rabbit, and various fishes—show a brilliant fluorescence when illuminated with ultraviolet light. When fresh bullock lenses were examined for fluorescence in the dark at room temperature, an intense blue emission was observed which had a lifetime shorter than 10^{-3} second, since no light was observed in our phosphoroscope which had this resolution time. If the lenses were permitted to attain temperature equilibrium at 194° K. (dry-ice temperature), an even more intense emission was exhibited, which persisted, with progressive fading, for about 20 seconds after the exciting light was removed. Such a long-lived afterglow and the shift from fluorescence to phosphorescence on cooling are characteristics of compounds which can undergo singlet-triplet intercombinations. To resolve the question whether the observed phosphorescence was emitted from a triplet state or by a crystal phosphor, we examined the decay kinetics and the activation energy of the emission. Emissions of light by crystal phosphors involve activation energies and are thus strongly temperature-dependent, while the emission decays from triplet excitation states require no activation energy and are temperature-independent. In addition,

the first-order decay kinetics characteristic of triplet emissions is not to be expected with crystal phosphor emissions.

The temperature dependence of the emission lifetime for bullock lens was first qualitatively studied in the following manner. Two Dewar vacuum flasks were placed side by side, one containing dry-ice freezing mixture, the other liquid nitrogen. A test tube holding the bullock lenses was immersed in each. After temperature equilibrium was established, the two test tubes were illuminated side by side for 2–3 seconds with ultraviolet light and then returned to their respective Dewar flasks, and the gradual disappearance of their emissions was observed. The afterglow faded out in both tubes at about the same time, although the temperature difference between the two systems exceeded 110° C. This temperature-independent lifetime strongly pleads for the assumption that the observed phosphorescence comes from a triplet excitation state.

These qualitative observations were quantitatively corroborated from kinetic studies carried out as described under the technique for Criterion 3, decay kinetics. Figure 1 depicts the results. It is to be noted first that the curves fit a first-order kinetic equation, i.e., the decay rate is a function only of the concentration of the residual metastable, triplet, species. Second, since the decay-rate constant is temperature-independent, the activation energy is negligible, and we have further evidence for triplet-state involvement.

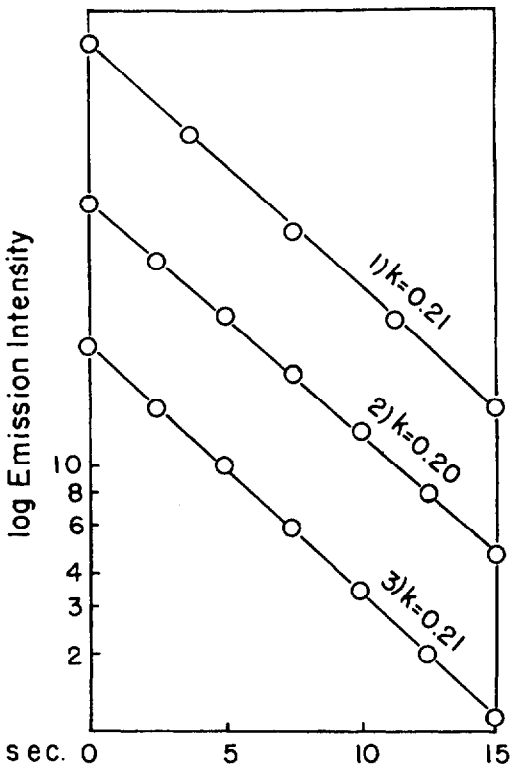

FIG. 1.—Phosphorescent decay kinetic data for lens systems. k = rate constant. *1*, intact ox lens, T = 194° K., emissions unfiltered; *2*, intact ox lens, T = 77° K., emission measured at 420 μ; *3*, homogenized ox lens (diluted 1 to 30 with water), T = 77° K., emission measured at 420 mμ.

The structural protein of the lens forms a fairly solid gel in vivo and can thus be expected to be fixed in space. This matrix should also impede the diffusion of dissolved protein molecules. This assumption must hold even more strongly for a frozen system. The change from fluorescence to phosphorescence with cooling can thus hardly be ascribed to dimerization or to higher polymerization of protein molecules but rather must be attributed to a change in the solvent and its relation to the protein.

If the lenses are minced in a Latapie mincer and suspended in 30 volumes of water (our lens homogenate), the suspension shows no striking fluorescence and no phosphorescence at 194° K. The same is true for less dilute suspensions. If, however, the suspensions are cooled further to 77° K., they emit a phosphorescence with an afterglow duration indistinguishable from that of the intact lens.

It was noted earlier that the addition of glucose to aqueous solutions of fluorescent substances in many instances enhances the phosphorescence of the frozen state.

especially at 194° K. This effect was ascribed to a more intimate contact of the luminescent species with water, glucose disfavoring a sharp separation of water crystals from the solute. This effect was observed also with the lens suspension. If the suspension contained 0.5 per cent glucose, freezing to 194° K., with subsequent illumination, caused the appearance of a strong blue emission resembling that of the intact lens and persisting for about half a minute after removal of the illumination. This afterglow was fairly independent of glucose concentration, being equally strong at 0.25 per cent and at 5 per cent. The light intensities were, under the conditions of our experiment, also independent, within wide limits, of the concentration of the suspension, the afterglow being about equally strong whether 1 part of lens was suspended in 30 or in 300 parts of water.

The fact that the material of the lens loses its fluorescence and phosphorescence by being minced and suspended in water indicates that the proteins in the native state of the lens are kept in a specific condition which favors these light emissions and the underlying excitations. This condition in all probability involves some specific relation to the solvent water, which relation can be restored by the addition of glucose.

The phosphorescence of the frozen (194° K.) lens suspension was strongly quenched if, in addition to the presence of 0.5 per cent glucose, the solution was made to 3 per cent (v/v) with glycerol or 0.25 per cent (v/v) with methanol prior to freezing. Acetone was found to be inactive even at a concentration of 8 volumes per cent. The phosphorescence was also insensitive to the action of oxygen. Equally inactive were classes of "quenchers" such as KI (10^{-3} M), NaSCN (10^{-3} M), 2,4-dinitrophenol (10^{-5} M), and 2,4-dinitronaphthol (5×10^{-6} M).

Figure 1 also gives the decay curve for homogenized lens phosphorescence at 77° K., which was no different at 194° K. In addition, it should be noted that at 77° K. the decay-rate constant for homogenized lens phosphorescence was the same without as with glucose.

The question arises whether the phosphorescence of the lens is due to some superstructure or to smaller units like amino acids making up the molecule. The paper of Debye and Edwards[4] appears to be the first systematic attempt to examine protein phosphorescence. They observed that most proteins emitted a brilliant blue phosphorescence at liquid-nitrogen temperature. The visible spectrum was found to be pH-dependent, the protein phosphorescence from an alkaline medium exhibiting a minor maximum at 418 mμ and an intense maximum at 440 mμ. In an acid medium the results were less consistent, the main maximum appearing at 417 mμ. The phosphorescent spectra of tyrosine and tryptophan were found to lie in the same spectral region as the protein phosphorescence. Debye and Edwards found the amino acid phosphorescent emission decays to be exponential and suggested that the exponential decay phosphorescence of proteins was due to their content of tyrosine, tryptophan, and possibly phenylalanine. From their finding that the phosphorescent decay was temperature-independent, they felt that an electronic transition was involved. Grossweiner,[5] using flash photolysis techniques, has complemented this work of Debye and Edwards and has presented spectra for the metastable states of ovalbumin, tyrosine, phenol, tryptophan, and indole. The wave-length shift, temperature independence, and metastable absorption spectra obtained by these workers suggested triplet-state involvement. Grossweiner further concluded that

the metastable absorption spectrum for ovalbumin principally represented the sum of contributions from tyrosine and tryptophan, with negligible effects from the peptide linkages.

The lens phosphorescence which we have observed (see Fig. 1) appears to have a slightly longer mean lifetime (5 seconds) than the protein phosphorescence studied by Debye and Edwards (approximately 3 seconds). In addition, we have observed slight differences in the kinetic data for amino acid phosphorescences when compared with the data of Debye and Edwards, but these are probably differences in degree only and not in kind arising from differences in technique.

2. AMINO ACIDS

The following amino acids were tested: *dl*-alanine, *l*-glutamic acid, *l*-histidine, *dl*-phenylalanine, *l*-tyrosine, and *dl*-tryptophan. None of these in aqueous solution showed any striking luminescence on visual observation under the ultraviolet lamp, frozen or unfrozen. Of these amino acids it might be anticipated from absorption spectral data that tryptophan and possibly tyrosine would be the only compounds which could possibly emit a visible fluorescence. This fact becomes apparent when one examines the fluorescent spectral data for the aromatic amino acids reported in the recent publications of Bowman, Caulfield, and Udenfriend,[6] Sprince, Rowley, and Jameson,[7] and Teale and Weber.[8] Phenylalanine and tyrosine emissions lie exclusively in the ultraviolet, while tryptophan emission, with a maximum in the near-ultraviolet, has a long-wave-length tail which "spills" over into the visible out to about 440 mμ. For this reason, therefore, a tryptophan solution of proper dilution, upon exposure to an unfiltered ultraviolet lamp, emits a distinct, though weak, violet fluorescence easily observable with the unaided eye. The failure to observe any appreciable fluorescence in a 0.01 M tryptophan solution, while a solution of 10^{-4} M emits a distinct violet light, indicates that the negative results at higher concentration may be due to self-quenching.

This emission picture changed markedly for the aromatic acids when the solutions were made to 0.5 per cent in glucose prior to freezing. Tyrosine and tryptophan in 10^{-3} molar concentration gave short and prolonged afterglows, respectively. These emissions were of considerable intensity. The behavior of tryptophan appeared similar to that of the lens, being blue and lasting for about half a minute. The wave-length shifts for these two compounds in going from fluorescence to phosphorescence were visually apparent, the colorless emission of tyrosine shifting to blue and the violet emission of tryptophan shifting to blue.

The marked enhancement of phosphorescence in the frozen state displayed by many compounds in the presence of glucose (other sugars were equally as effective) motivated us to examine this effect in more detail. Studies were made with tryptophan as the luminous species.

It was found that the phosphorescent decay-rate constant for tryptophan-glucose systems in ice was practically independent of temperature[9] and tryptophan concentration. In addition, we have observed an influence of glucose concentration on the phosphorescent emission intensity. When the tryptophan concentration was held constant and the glucose concentration varied, the emission intensity was observed to pass through a maximum, while the decay-rate constant remained essentially unchanged. A preliminary examination of this effect made with a tryptophan con-

centration of 5×10^{-4} molar resulted in a maximum phosphorescent intensity at a glucose concentration of 0.27 per cent. Table 1 summarizes the results for the slight influence of temperature and tryptophan concentration on the phosphorescent decay kinetics.

Substitution of the rate data from Table 1 for the 10^{-3} molar tryptophan system into the equation,

$$\log \frac{k_2}{k_1} = \frac{\Delta H_a}{2.303R}\left(\frac{T_2 - T_1}{T_2 T_1}\right),$$

gave an activation energy of only 76 calories. In this equation k_2 and k_1 are the rate constants at the high (T_2) and low (T_1) temperatures, respectively; ΔH_a is the activation energy; and R is the gas constant.

Figure 2 illustrates the influence of temperature on the phosphorescent decay-rate constant for a 10^{-4} molar tryptophan concentration system containing 1 per cent glucose. It should be observed that the tryptophan phosphorescence was insensitive to oxygen.

TABLE 1

THE INFLUENCE OF TEMPERATURE AND TRYPTOPHAN CONCENTRATION ON THE DECAY-CONSTANT FOR TRYPTOPHAN PHOSPHORESCENCE*

TRYPTOPHAN CONCENTRATION (MOLES PER LITER)	—RATE CONSTANT, k— 194° K.	77° K.	TRYPTOPHAN CONCENTRATION (MOLES PER LITER)	—RATE CONSTANT, k— 194° K.	77° K.
5×10^{-3}	0.40	0.39	10^{-5}	0.26	0.17
10^{-3}	0.27	0.20	10^{-6}	0.24	0.17
10^{-4}	0.21	0.18			

* The glucose concentration was held constant at 0.25 per cent. Rate data were obtained with the spectrophotometer set at 420 $m\mu$ and with 2.0-mm. slit widths. Systems were contained in 25 × 200-mm. Pyrex test tubes and, following a minimum of 15 minutes at the proper temperature to allow temperature equilibration, were illuminated with the unfiltered beam of a high-pressure Hanovia mercury arc for 5 seconds.

Figure 3 contains the phosphorescent decay kinetic data of the tyrosine-glucose system. The intensity of the emitted light in the absence of glucose was too low to permit its measurement by the technique used in this study. At 194° K., even in the presence of glucose, the intensity of the emitted light was so low that it was difficult to obtain a satisfactory decay curve, and the reported rate constant should be considered accurate within ±5 per cent. We have made no effort to obtain kinetic data for phenylalanine.

Wave-length shifts, long-lived afterglows, first-order decay kinetics, and negligible temperature dependence of the rate constants may be presented as presumptive evidence that tyrosine and tryptophan afterglows are emitted from a metastable triplet state.

3. ADENINE, ADENOSINE, ADENOSINE PHOSPHATE, ADENOSINE DIPHOSPHATE, AND ADENOSINE TRIPHOSPHATE (ATP)

Euler, Brandt, and Neumüller[10] and Stimson and Reuter[11] presented fluorescent data for several purines and pyrimidines and their derivatives and gave the color of their emissions as a function of pH. Since we have been interested in the energy configuration of ATP in relation to its possible functional participation in the energy transformations in which the molecule participates, we have limited our study to the purine adenine and its derivatives.

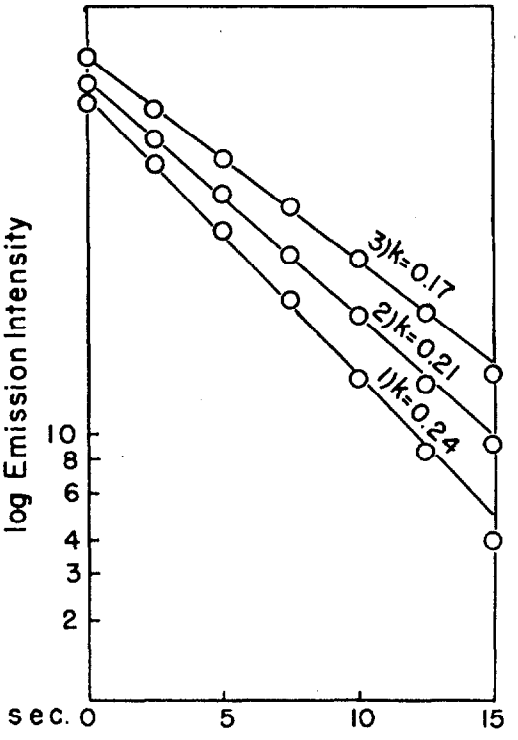

FIG. 2.—Influence of temperature on the phosphorescent decay rate constant, k, for tryptophan-glucose system; tryptophan = 10^{-4} M, glucose = 1 per cent. *1*, system *approaching* temperature equilibrium at 194° K.; *2*, system at temperature equilibrium at 194° K.; *3*, system at temperature equilibrium at 77° K., emission measured at 420 mμ.

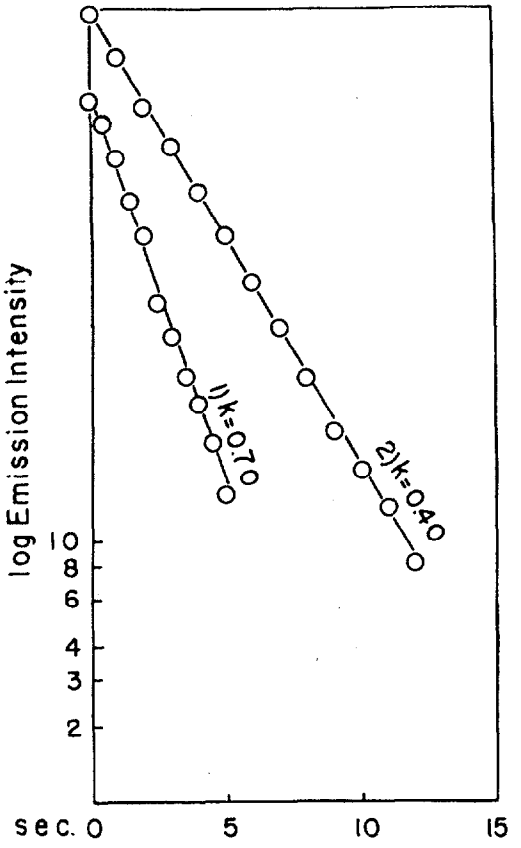

FIG. 3.—Phosphorescent decay kinetic data for tyrosine-glucose systems. k = rate constant. *1*, 10^{-3} M tyrosine + 0.5 per cent glucose, T = 194° K., emission unfiltered; *2*, 4.4×10^{-4} M tyrosine + 0.3 per cent glucose, T = 77° K., emission measured at 420 mμ.

In view of the fact that the absorption bands of the adenine compounds lie rather deep in the ultraviolet, it might be anticipated that only slight or no visible fluorescence would be observable from them upon proper excitation. Stimson and Reuter[11] reported a faint green fluorescence for adenine sulfate in strong alkali and a faint blue emission in weak alkali. They observed no emission for adenylic acid solutions at any pH. In our work we have never observed a visible emission from adenine, adenosine, or any of the phosphate derivatives at any pH at room temperature. Adenine compounds of 0.01–0.0005 M concentrations, when frozen to 194° K., displayed a very faint blue short-lived afterglow. This emission could usually be enhanced in intensity by the addition of glucose to 0.5 per cent prior to freezing. ATP, for example, with glucose added, displayed an intense afterglow lasting for about 15 seconds after the exciting light was removed. ATP, in contrast to tryptophan, displayed no self-quenching in 0.01 M concentration. This may have biological significance, especially in muscle, where ATP exists in rather high concentration. While the ATP phosphorescence was insensitive to KI, NaSCN, 2,4-dinitrophenol, 2,4-dinitronaphthol, and acetone (8 per cent v/v) it was abolished by 5 per cent (v/v) glycerol and 0.1 per cent (v/v) methanol at 194° K. These later quenching effects were not observed, however, at 77° K.

Figure 4 summarizes the phosphorescent decay kinetics for all the adenine systems studied. Kinetic data at 194° K. were obtained for ATP only and indicated that the rate constant was practically independent of temperature. Though the emission intensities were found to be markedly sensitive to pH, the rate constants remained essentially independent of this variable. An important feature observed in studies of pH effects on the emission intensities was the finding that the amino nitrogen at position 6 on the adenine ring must be dissociated for luminescence to occur. Varying the pH of adenine-compound systems from 2 to 7 by units of 1 gave a progressive increase in the phosphorescent intensity from nil to brilliant. Since, according to the work of Alberty, Smith, and Bock,[12] the amino group has a pK_a of approximately 4, we visualize the increased alkalinity as dissociating the "adeninium" cation. This dependence of luminous intensity on the degree of dissociation of an amino group is not a unique finding. Ley and von Engelhardt[13] noted that, whereas the aniline molecule was fluorescent, the anilinium cation was nonfluorescent. By contrast, Ley and Gräfe[14] and Dickson[15] found that the blue fluorescence of alpha- and beta-naphthylamine was not lost by proton addition but, instead, was shifted into the ultraviolet, with, however, a considerable diminution in intensity. Lewis and Kasha[16] have given phosphorescent emission data for aniline and alpha- and beta-naphthylamine, but we have found no reports on the phosphorescence of the anilinium cation or of the alpha- or beta-naphthylammonium cations.

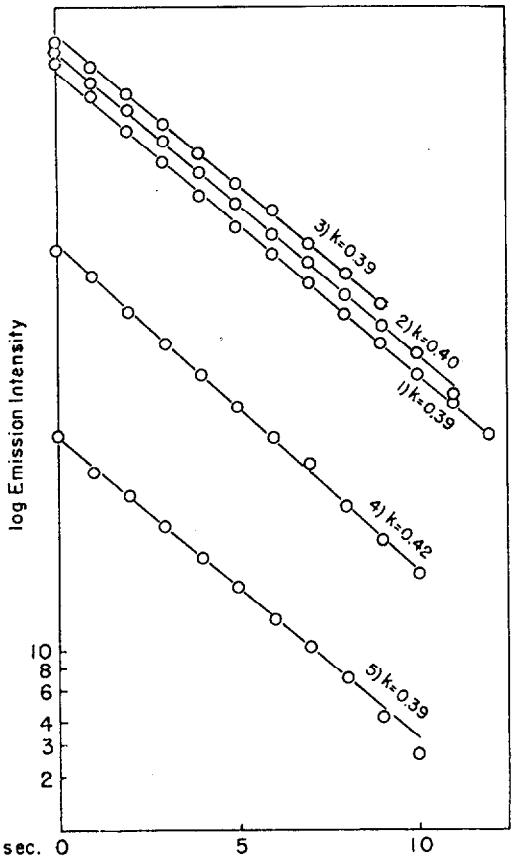

FIG. 4.—Phosphorescent decay kinetic data for adenine (*1*), adenosine (*2*), adenosine-5′-phosphate (*3*), adenosine-5′-diphosphate (*4*), and adenosine-5′-triphosphate (*5*). All concentrations 0.001 *M*; pH's adjusted to 5 with acetate buffer prior to freezing. T = 77° K. All emissions measured at 420 mμ. k = rate constant.

For the adenine derivatives studied, therefore, the wave-length shifts, i.e., from no visible emission to an intense blue afterglow, the long lifetimes, and the first-order kinetics plead for triplet-state participation in the emissions observed for these compounds.

4. NUCLEIC ACIDS

Nucleic acids show a long-lived phosphorescence which might be due to the collective action of the column of cyclic structures constituting, according to the model of Watson and Crick,[17] the core of the molecule. The central cyclic compounds would thus, according to this assumption, be analogous to Scheibe's pseudoisocyanine dyes,[18] which polymerize to columns in the shape of "money-rolls" through which excitons are freely propagated by resonance.

The phosphorescent light emission from aqueous solutions of DNA and RNA preparations, 0.1–1.0 per cent, frozen to 194° K., could be prolonged by the addition of 0.25–4 per cent glucose. This prolongation was abolished by small concentrations of glycerol (3 per cent, v/v) or methanol (0.25 per cent, v/v), while acetone (16 per cent, v/v) was found to be inactive. The phosphorescence was found to be insensitive to KI, 2,4-dinitrophenol, 2,4-dinitronaphthol, salicylate (0.002 M), or pyrrolazote (2×10^{-4} M). Morphia and aconitine (10^{-3} M) were equally inactive, while colchicine in 2×10^{-4} M concentration quenched DNA strongly, the effect increasing somewhat on storage. Since the nucleic acids were present in rather high concentration, 0.5–1 per cent, there was a disproportionality between the molarity of the alkaloid and the mass of the nucleic acids or the number of nucleotides building the nucleic acid molecule. This pleads for a collective action of the nucleotides in the production of phosphorescence. Scheibe[19] found the energy transmission in his dye polymers decreased by relatively small concentrations of hydroquinone, the conduction within the "money-rolls" being cut by the interposition of a "dud," a hydroquinone molecule. Hydroquinone at 0.001 M also strongly quenched the phosphorescence of DNA. The first preliminary experiments repeated with high-polymer DNA indicated that the quenching action of colchicine was about ten times stronger with this material than with the low-polymer DNA, the alkaloid being active in 10^{-5} M concentration. This concentration is near the level which is effective in vivo. If the alkaloid acts by cutting the energy transmission through the row of central bases, then it should be more active the longer the molecule.

The carcinogen benzpyrene also showed a fairly strong quenching activity. In these experiments the carcinogen was dissolved in acetone (0.1 per cent). One-tenth of a milliliter of this solution added to 5 ml. of 0.5 per cent DNA almost completely eliminated the long afterglow enhanced by glucose. In this instance, too, the action seemed to be more marked when high-polymer DNA was used in the experiment. Benzpyrene, as is generally known from the work of Brock, Druckrey, and Hamperl,[20] emits a blue light when molecularly dispersed and a yellow-green light in colloidal emulsion when illuminated by ultraviolet light. This can be strikingly demonstrated by adding a drop of acetone solution of benzpyrene to water. The resulting colloidal solution emits a yellow-green fluorescence which changes dramatically to blue if the solution is shaken up with a little solid caffein. The purine forms a complex with the carcinogen, bringing it into molecular dispersion. Analogous behavior can be demonstrated with DNA, indicating that the carcinogen complexes with the bases of the nucleic acids. This might have been anticipated also from the work of Booth and Boyland,[21] who demonstrated that the variably carcinogenic bicyclic dibenzacridines formed well-defined crystalline complexes with purines and nucleic acids.

Figure 5 depicts the phosphorescent decay kinetic results as measured for DNA and RNA at 77° K. Decay kinetics are not reported for these compounds at 194°K., for the emission intensities were too low to be recorded by the technique here employed. The rate constants given should be considered only as indicating the order of magnitude of the decay rate, for the purity of these preparations was uncertain. The rate constant for DNA appears to be complex, and until highly purified preparations of reasonably well-known polymer composition are avail-

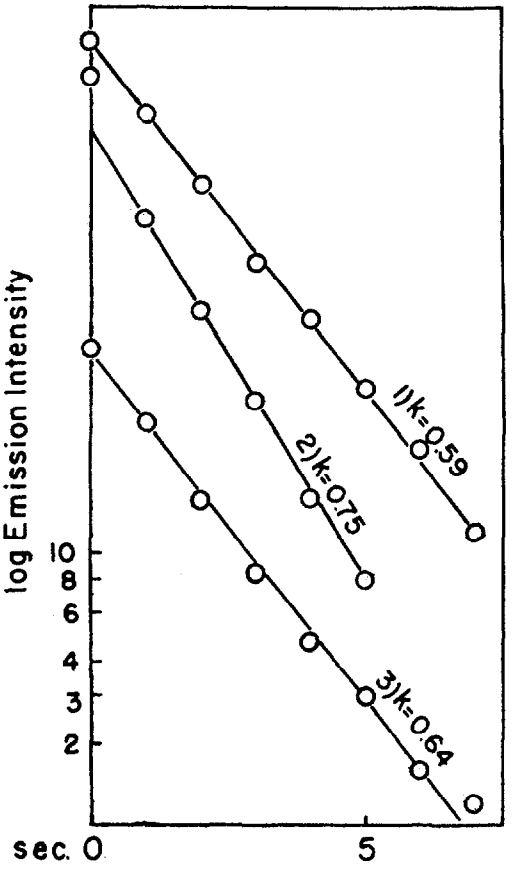

FIG. 5.—Phosphorescent decay kinetic data for desoxyribonucleic acid and ribonucleic acid. $T = 77°$ K. k = rate constant. *1*, 1 per cent DNA + 0.25 per cent glucose, 480 μ; *2*, 1 per cent DNA, no glucose, 520 mμ; *3*, 1 per cent RNA + 0.25 per cent glucose, 400 mμ.

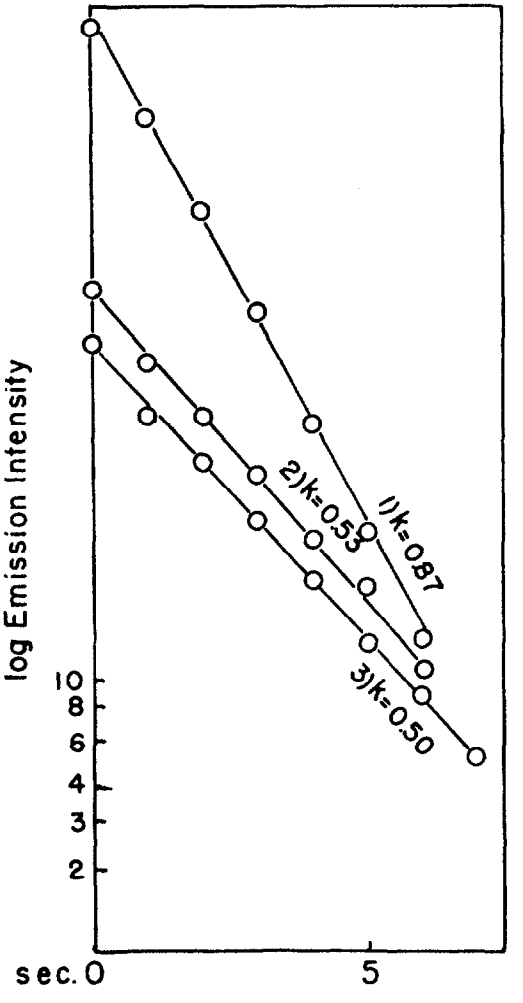

FIG. 6.—Phosphorescent decay kinetic data. *1*, quinidine-SO_4, 10^{-4} M aqueous solution + 0.25 per cent glucose, green emission measured at 510 mμ, $T = 77°$ K.; *2*, progesterone, 7.3×10^{-4} M in 95 per cent ethanol + 0.5 per cent glucose, blue emission measured at 420 mμ, $T = 77°$ K.; *3*, alpha-estradiol, 10^{-3} M in 95 per cent ethanol, no glucose, violet emission measured at 410 mμ, $T = 77°$ K. k = rate constant.

able, we will restrict our comments to one observation, namely, that while a 0.1 per cent DNA solution (glucose present) displayed a blue phosphorescence, a 1 per cent DNA solution (also in the presence of glucose) gave a green phosphorescence. We have noticed a somewhat analogous phenomenon with adenosine. Whereas adenosine in the absence of glucose emitted a distinct green phosphorescence, in the presence of glucose the emission reverted to the blue characteristic for adenine and the adenosine phosphate derivatives. The effect was most marked at low pH values, and none of the other adenine compounds showed the effect. Therefore, the difference observed in the rate constant for DNA may reflect a mixture of these two emissions. The data predict that the green emitting system, if real, will be found to have the higher rate constant. No such wave-length shifts have been observed for RNA.

First-order kinetics and increased lifetimes are the only criteria we have obtained so far for the nucleic acids which suggest triplet-state involvement.

5. MISCELLANEOUS

Figure 6 contains rate data for quinidine sulfate, progesterone, and alpha-estradiol phosphorescences, all measured at 77° K. and at the wave lengths given. Quinine sulfate gave practically the same rate constant as quinidine sulfate, and glucose addition was necessary for both compounds in order to obtain sufficient luminous intensity to permit decay curves to be run. The emission was a distinct green in both instances and of rather intense brightness, though of very short duration. The blue emission of progesterone and the violet emission of alpha-estradiol, though of low intensities, could both be measured without glucose addition; in fact, glucose addition had little influence on their emission intensities. It should be noted that the solvent for the hormones was 95 per cent ethanol, not water. The very low emission intensities at 194° prevented rate-constant measurements by the technique used in this study.

Quinine and quinidine sulfate fluoresced blue, while the phosphorescence was green. We have measured the fluorescence of alpha-estradiol (unpublished) and found it to be exclusively ultraviolet, with a maximum at approximately 310 μ. We were unable to obtain any indication of progesterone fluorescence. With these compounds, therefore, wave-length shift (with the possible exception of progesterone), first-order kinetics, and increased lifetimes represent the criteria suggestive of triplet-state involvement.

6. DYES

In Figure 7 are presented a few preliminary kinetic studies made for the dyes acridine yellow and acridine orange. The break in rate curves at the higher dye concentration and elevated water content suggest that polymer involvement may influence the rate constant. Zanker[22] has made a detailed study on the influence of solvent, concentration, temperature, and pH on the absorption and emission characteristics of acridine orange that may prove to be pertinent here.

We have measured the following emission shifts for these two dyes in going from fluorescence to the phosphorescent state. Acridine yellow fluoresced at 511 mμ and phosphoresced with a maximum at 614 mμ. Acridine orange gave a fluorescence band with a maximum at 530 mμ and a phosphorescence-band maximum at 660 mμ. These wave-length shifts, first-order kinetics, and long-lived afterglow, we cite, therefore, as evidence for triplet-state participation in the phosphorescence of these dyes.

7. WATER

A finding which we can mention only briefly at this time was an observation concerning the emission from ice following ultraviolet excitation.[23] The "afterglow" was not visible in an undarkened room, and we have made no attempt to visualize it with dark-adapted eyes. The intensity appeared greatest when the emission from ice at 77° K. was permitted to impinge unfiltered directly onto the RCA 1P28 photomultiplier tube set electronically at maximum sensitivity. Precautions were always taken to insure that this ice emission did not interfere with the

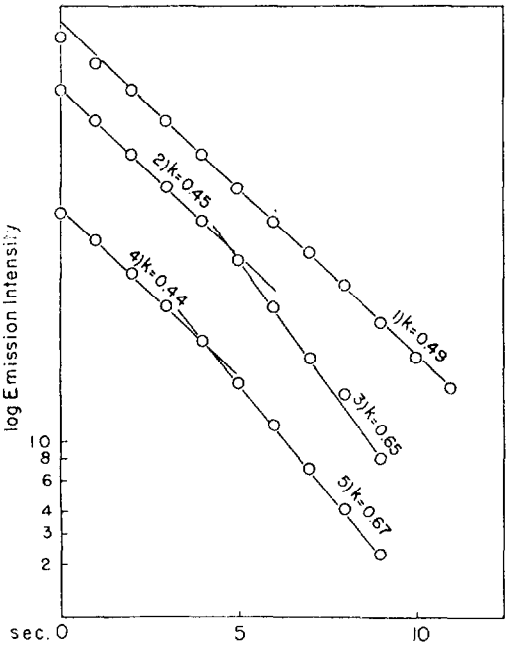

FIG. 7.—Phosphorescent decay kinetic data for the dyes acridine yellow and acridine orange. T = 77° K. k = rate constant. *1*, 10^{-4} M acridine yellow in 90 per cent ethanol + 10 per cent water (v/v), emission measured at 550 mμ; *2–3*, 5×10^{-4} M acridine yellow in 50 per cent ethanol + 50 per cent water (v/v), emission measured at 530 mμ; *4–5*, 5×10^{-4} M acridine orange in 50 per cent ethanol + 50 per cent water (v/v), emission measured at 600 mμ.

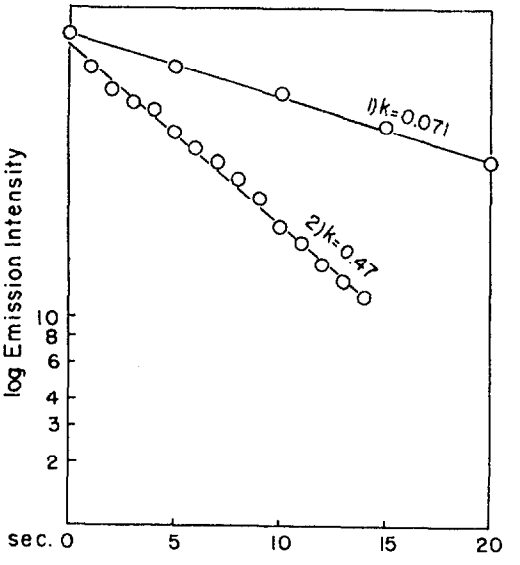

FIG. 8.—Phosphorescent decay kinetics for the unfiltered afterglow from ice at 77° and 194° K. The ice was frozen around the outside of a hollow aluminum bar (freezing mixtures inside) and exposed for 5 seconds to the unfiltered beam of a high-pressure mercury lamp prior to the kinetic run. k = rate constant. 1 = 77° K., 2 = 194° K.

kinetic measurements. Rate data for this emission at 77° and 194° K. are given in Figure 8. These results were fairly reproducible. It is interesting that the decay appears to obey first-order kinetics and has a rather low activation energy. Unfortunately, the intensities were too low to permit us to obtain reliable information about their spectral distribution. The rate constant, however, appeared to be about the same whether the emission was permitted to traverse a Corning 5860 band-pass filter (maxinum wave length passed at 360 mμ) or a Corning 3486 cutoff filter, cutting out light *below* 510 mμ.

DISCUSSION AND SUMMARY

Fluorescent and phosphorescent spectral data, kinetic studies (including order of reaction), lifetimes, and activation-energy considerations have been presented for a wide variety of biological compounds and the dyes acridine yellow and acridine orange. Results were obtained which we submit as evidence that electronic transitions involving singlet-triplet intercombinations are functional in these compounds.

The marked optical changes observed in freezing *aqueous* solutions, namely, the shift from fluorescence to phosphorescence, appear to be a universal property of fluorescent materials.

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[9] The change in the rate constant never varied by more than a factor of 2 for a change in the temperature of more than 100° C. Consequently, the activation energy for the tryptophan-glucose system may be considered negligible. It was found, nonetheless, that slight variations in the rate constant could be controlled when more care was exercised in controlling the temperature (e.g., insuring that temperature equilibrium was established) and tryptophan concentration.

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[23] Leonard I. Grossweiner and Max S. Matheson (*J. Chem. Phys.*, **20**, 1654–1655, 1952; **22**, 1514–1526, 1954) have reported observing an X-ray-induced fluorescence from ice at low temperatures. The emission at −183° C. had an almost symmetrical band with a peak at 385 μ. They observed no phosphorescence whatever. That energy was trapped, however, was made evident from glow-curve studies following X-ray excitation. The difference between their activation energy (0.25 ev.) and ours (0.02 ev.) indicates that different mechanisms are involved in "trapping" the energy. This would be expected, since energy was stored in the one instance by X-ray excitation and in the other instance by ultraviolet light. We have made no study of the influence of oxygen on the emission, and dissolved oxygen was not excluded prior to freezing. Grossweiner and Matheson observed a suppression of thermoluminescence by oxygen.